

AD _____

CONTRACT NO: DAMD17-94-C-4044

TITLE: Molecular Biological Approaches to Studying Cyanide
Detoxification by Rhodanese

PRINCIPAL INVESTIGATOR(S): Thomas P. Dooley, Ph.D.

CONTRACTING ORGANIZATION: Southwest Foundation for
Biomedical Research
San Antonio, Texas 78228-0147

REPORT DATE: January 1996

TYPE OF REPORT: Final

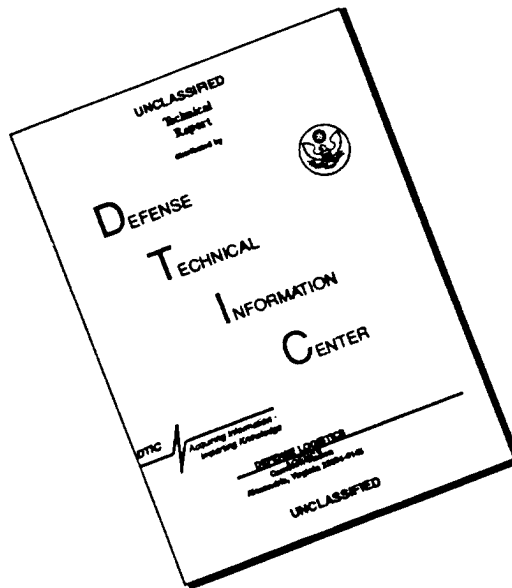
PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution
unlimited.

The views, opinions and/or findings contained in this report are those
of the author(s) and should not be construed as an official Department
of the Army position, policy or decision unless so designated by other
documentation.

19960419 164

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE January 1996		3. REPORT TYPE AND DATES COVERED Final (16 May 94 - 15 Dec 95)
4. TITLE AND SUBTITLE Molecular Biological Approaches to Studying Cyanide Detoxification by Rhodanese			5. FUNDING NUMBERS DAMD17-94-C-4044	
6. AUTHOR(S) Thomas P. Dooley, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Southwest Foundation for Biomedical Research San Antonio, Texas 78284			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Rhodanese (thiosulfate sulfurtransferase) is expressed as high levels in liver and is involved in the detoxification of cyanide. The full-length cDNA corresponding to the mouse rhodanese gene (<i>Tst</i>), which is located on chromosome 15, was cloned by PCR amplification of a liver cDNA library and subjected to DNA sequencing. Alignment of the rhodanese cDNA sequences from mouse and rat, which we previously cloned (Biochem. J. 275:227-231), revealed 97.3 percent identity at the protein level and 94.6 percent identity at the DNA level. When the mouse and rat cDNAs were expressed under the control of IPTG-inducible promoters in <i>E. Coli</i> , the cell extracts exhibited cyanide-metabolizing activity, indicating that both genes encode functional rhodanese molecules. The results of the study were published by Dooley et al. in Biochemical and Biophysical Research Communications 216:1101-1109 (1995).				
14. SUBJECT TERMS cyanide, rhodanese, cDNA cloning, DNA sequencing, recombinant protein, prophylactic agent, therapeutic agent			15. NUMBER OF PAGES 18	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to *stay within the lines* to meet *optical scanning requirements*.

Block 1. Agency Use Only (Leave blank).

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C - Contract	PR - Project
G - Grant	TA - Task
PE - Program Element	WU - Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. (If known)

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

DOD - See DoDD 5230.24, "Distribution Statements on Technical Documents."

DOE - See authorities.

NASA - See Handbook NHB 2200.2.

NTIS - Leave blank.

Block 12b. Distribution Code.

DOD - Leave blank.

DOE - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.

NASA - Leave blank.

NTIS - Leave blank.

Block 13. Abstract. Include a brief (*Maximum 200 words*) factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code (*NTIS only*).

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

TPD Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

TPD In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

TPD In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

TPD In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Thomas P. Dooly 3 Jan. 96
PI - Signature Date

Table of Contents:

<u>Section:</u>	<u>Pages</u>
Table of Contents	4
Introduction	5-6; and Appendix 1101-1102
Body	6; and Appendix 1102-1107
Conclusions	6; and Appendix 1107-1108
References	6; and Appendix 1108-1109
Appendix:	
Additional Unpublished Research Accomplishments:	7-8
-I. Mouse Rhodanese Genomic Clone	7
-II. Chromosomal Mapping in Mouse	7
-III. Construction of Mammalian cDNA	7-8
Expression Constructs	
-IV. Surfactant Promoter Clone	8
General Comments:	
-Personal Visits between Dr. Dooley and	8
USAMRICD Representatives	
-Press Releases	8-9
-Dr. Dooley's Move to Birmingham	9
Publication Resulting from Contract	9; and Appendix 1101-1109

Introduction: Hydrogen cyanide is an effective and inexpensive asphyxiant chemical weapon, for which no prophylactic agents are currently available. Cyanide causes injury by inhibition of cytochrome oxidase, which is involved in electron transport and cellular respiration. The liver enzyme rhodanese (thiosulfate sulfurtransferase) is capable of catalyzing *in vitro* the conversion of cyanide to the less toxic molecule thiocyanate. We had previously isolated the cDNA encoding this gene from rat. Since rhodanese can detoxify cyanide, we hypothesized that cloned recombinant rhodanese molecules could serve as biologic prophylactic and therapeutic agents. In order to test this hypothesis, we had proposed a series of related studies (for a 3 year period) to demonstrate cyanide-metabolizing activity of a cloned rodent (either rat or mouse) recombinant enzyme, and to develop transgenic mouse models that overexpressed rhodanese as a proof of concept that elevated endogenous levels of rhodanese could in fact enhance resistance to cyanide.

The cumulative results obtained to date (representing 18 months of effort) on this contract (excluding the "Additional Research Accomplishments" section below) were published on 22 November 1995 in Biochemical and Biophysical Research Communications (see Appendix). The article is entitled "Mouse Rhodanese Gene (*Tst*): cDNA Cloning, Sequencing, and Recombinant Protein Expression" by T.P. Dooley, S. Nair, R.E. Garcia I.V., and B.C. Courtney. This report includes a concise representation of: (1) cDNA cloning and DNA sequencing of mouse rhodanese; (2) amino acid sequence comparisons between mouse, rat, and cow rhodanese enzymes; and (3) demonstration of biochemical activity of recombinant mouse and rat rhodanese expressed in bacteria. The Introduction section of this publication provides full details concerning the nature of the problem and background information. *Refer to the Introduction section of Dooley et al. (1995), pages 1101-1102, for further details.*

The subcontract to Ohio State University to generate transgenic mice was not commenced during the first 18 month period, as originally proposed. We encountered unforeseen difficulties in demonstrating activity of the cloned rat rhodanese cDNA, as outlined in the original Statement of Work (SOW). In view of these initial difficulties, we chose to clone the mouse rhodanese cDNA, as this option was permitted under the approved SOW, and would further strengthen the study, albeit resulting in an intentional delay in commencing the transgenic mouse component. Once the mouse *Tst* cDNA was cloned and sequenced we prepared bacterial expression vectors to express it. These studies revealed to us the cause of the lack of apparent activity by the rat clone. We were then able to address this problem,

and in fact were successful at demonstrating activity by both the mouse and rat recombinant proteins.

Body: *Refer to the Materials & Methods and Results sections of Dooley et al. (1995), pages 1102-1107.*

Conclusions: *Refer to the Discussion section of Dooley et al. (1995), pages 1107-1108.* If the project is resumed at Southern Research Institute, as requested by Dr. Dooley, we should be able to actively pursue the transgenic mouse component. Some suggestions for improvement of the study have been made collectively by Dr. Dooley, Cpt. B. Courtney (USAMRICD), and Dr. S. Baskin (USAMRICD), including: (1) One of the transgene constructs should be designed to express rhodanese in various tissues and not be tissue specific. Several constructs of this type have already been prepared; (2) An effort should be made to determine the sequence of a natural intron within the mouse rhodanese gene, if one exists. Inclusion of an intron in the transgene construct might increase expression of rhodanese. We have several putative genomic clones ready for analysis by PCR; and (3) The recombinant rodent rhodanese protein(s) should be assayed *in vitro* with several of the thiosulfonate cofactors, which have already been tested at USAMRICD against non-recombinant purified mammalian enzymes.

References: *Refer to the References section of Dooley et al. (1995), pages 1108-1109.*

Appendix:

Additional Unpublished Research Accomplishments:

I. Mouse Rhodanese Genomic Clone: We have obtained several putative genomic clones of the mouse rhodanese gene by conventional library screening using the labeled mouse cDNA as a hybridization probe. The putative clones were isolated via a commercial source (Research Genetics, Huntsville AL). The putative genomic copy should permit us to identify via PCR amplification a natural intron within the *Tst* gene, to be potentially included within transgenic constructs. Inclusion of an intron is believed to increase the expression potential of transgenes *in vivo*.

Although beyond the scope of this contract, we could utilize the rhodanese genomic clone to develop a strategy to create a genetic rhodanese deficiency in mice (i.e., a "gene knock out"). A rhodanese-deficient mouse model would yield insights into the role of this enzyme in embryonic development and in cyanide metabolism. A rhodanese null mouse model is expected to be highly sensitive to cyanide, if in fact rhodanese is the central enzyme involved in cyanide metabolism as hypothesized.

II. Chromosomal Mapping in Mouse: We had previously published that the rhodanese gene is localized to mouse chromosome 15 [Leiter, E.H., Chapman, H.D., and Dooley, T.P. (1993) *Mouse Genome* 91:567-568]. We have continued a collaboration with Dr. Ed Leiter of the Jackson Laboratory (Bar Harbor, Maine) to further refine the mapping coordinates of this gene. We have determined by Southern blots using interspecific mouse backcross panels that the mouse *Tst* gene maps to mouse chromosome 15, circa 45-46 cM, in a region homologous to human chromosome 22q. The refined mapping results were published in an *erratum* in *Mouse Genome* 93:863 (1995), and are mentioned in the Discussion section in the Dooley et al. (1995) article (see attachment).

III. Construction of Mammalian cDNA Expression Constructs: The full-length mouse rhodanese cDNA fragment (from clone pTM1193) was subcloned by ligation into four mammalian cDNA expression constructs (pIRV-SV, pBabeNeo, pCR-3, and pCI-Neo). Two of these vectors are retroviral provirus constructs (pIRV-SV and pBabeNeo), which could potentially serve as

retroviral gene therapy agents following viral packaging. Plasmid DNAs from these vectors with and without the appropriate rhodanese cDNA insert may be used in calcium phosphate-mediated transfections into mammalian cell cultures to evaluate the expression of rhodanese from each chimeric construct. Furthermore, the constructs may be used in transgenic animals, although the pattern of gene expression is not expected to be tissue specific. Initial attempts at demonstrating activity in transfected Hela cells were not successful for unknown reasons, and sufficient time was not available during the 6th Quarter to resolve the issue.

IV. Surfactant Promoter Clone: We have cloned by PCR a genomic fragment of the human surfactant-C promoter for use in transgenic constructs. The SP-C promoter is known to be expressed at high levels in lung, and may serve well for expression of rhodanese in lung in transgenic mice.

General Comments:

Personal Visits between Dr. Dooley and USAMRICD Representatives:

(1) On 3 October 1994, Dr. Dooley met with Cpt. B. Courtney at the USAMRICD at Edgewood and presented a seminar entitled "Molecular Biology of Mammalian Rhodanese (Cyanide Sulfurtransferase) and Phenol Sulfotransferase Genes".

(2) Cpt. B. Courtney performed a site visit of Dr. Dooley's laboratory in San Antonio on 6 March 1995.

(3) On 14 July 1995, Dr. Dooley visited the USAMRICD at Edgewood to discuss research progress, and to present a general lecture entitled "The Basics of Gene Therapy".

Press Releases: A press release approved by the USAMICD was distributed to various media agents. This contract was cited in various articles, including the following examples:

(1) San Antonio Express News, Thursday 9 February 1995, page 2B, entitled "Biologist tracks

cyanide vaccine with Army grant”.

(2) San Antonio Medical Gazette, 15-21 February 1995, pages 6-7, entitled “Scientists’ cyanide-fighting recombinant protein may prove beneficial for armed forces/firefighters”.

(3) Austin (TX) American-Statesman on February 16, 1995, entitled “DNA research may help protect firefighters, soldiers from cyanide”.

(4) The Stars and Stripes, 27 February 1995, entitled “DNA Research Could Lead to Battlefield Antidote for Cyanide Poisoning”.

Dr. Dooley’s Move to Birmingham: On 15 November 1995, Dr. Dooley moved to Birmingham, Alabama as the Alabama Power Endowed Chair and Director of Molecular Pharmacology at Southern Research Institute. Coincident with the move, all research activities were terminated at the Southwest Foundation for Biomedical Research in San Antonio.

Publication Resulting from Contract (see attachment): T.P. Dooley, S. Nair, R.E. Garcia I.V., and B.C. Courtney (1995) “Mouse Rhodanese Gene (Tst): cDNA Cloning, Sequencing, and Recombinant Protein Expression” Biochemical and Biophysical Research Communications 216:1101-1109.

MOUSE RHODANESE GENE (*Tst*): cDNA CLONING, SEQUENCING, AND RECOMBINANT PROTEIN EXPRESSION

Thomas P. Dooley^{1*}, Shalini K. Nair¹, Roy E. Garcia IV¹, and Bernard C. Courtney²

¹Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX

²Applied Pharmacology Branch, U.S. Army Medical Research Institute of Chemical Defense,
Aberdeen Proving Ground, MD

Received October 27, 1995

SUMMARY: Rhodanese (thiosulfate sulfurtransferase) is expressed at high levels in liver and is involved in the detoxification of cyanide. The full-length cDNA corresponding to the mouse rhodanese gene (*Tst*), which is located on chromosome 15, was cloned by PCR amplification of a liver cDNA library and subjected to DNA sequencing. Alignment of the rhodanese cDNA sequences from mouse and rat, which we previously cloned (Biochem. J. 275:227-231), revealed 97.3 percent identity at the protein level and 94.6 percent identity at the DNA level. When the mouse and rat cDNAs were expressed under the control of IPTG-inducible promoters in *E. coli*, the cell extracts exhibited cyanide-metabolizing activity, indicating that both genes encode functional rhodanese molecules. © 1995 Academic Press, Inc.

INTRODUCTION: Rhodanese (EC 2.8.1.1; thiosulfate sulfurtransferase) is capable of enzymatically inactivating cyanide (CN⁻) *in vitro* by conversion into thiocyanate (SCN⁻), which is much less toxic (1, 2). This reaction *in vitro* involves the transfer of a sulfur atom from a sulfur donor cofactor, such as sodium thiosulfate, to the substrate. At least one other mammalian enzyme, 3-mercaptopyruvate sulfurtransferase can also catalyze the conversion of CN⁻ to SCN⁻ (3, 4). However, it is generally accepted that the systemic cyanide is detoxified within the liver and perhaps other tissues by rhodanese. Additional *in vivo* biochemical role(s) for rhodanese have been suggested, including the formation of iron-sulfur complexes (5), although this hypothesis has been challenged (6).

The evidence in favor of rhodanese playing a direct role *in vivo* in the detoxification of cyanide and cyanogenic agents include the following: (a) Rhodanese can enzymatically inactivate cyanide *in vitro* producing thiocyanate, and this metabolite is also formed *in vivo* when exposed to

* Corresponding author and reprint requests (current address): Thomas P. Dooley, Ph.D., Director of Molecular Pharmacology, Southern Research Institute, 2000 Ninth Ave. S., Birmingham, AL 35255. Fax: (205) 581-2877.

Abbreviations used: bp (base pairs); cDNA (complementary DNA); *Col2a1* (type II collagen alpha 1 gene); CN⁻ (cyanide ion); IPTG (isopropylthio-β-D-galactopyranoside); kb (kilobase); LB (L-broth medium); ORF (open reading frame); PCR (polymerase chain reaction); pI (isoelectric point); PMSF (phenylmethylsulfonylfluoride); SCN⁻ (thiocyanate ion); SDS (sodium dodecyl sulfate); *Tst* (thiosulfate sulfurtransferase / rhodanese gene).

cyanide; (b) Thiosulfate ion (among other agents) can serve as a sulfane sulfur donor for rhodanese *in vitro*, and intravenous injection of sodium thiosulfate aids in the biochemical detoxification of cyanide (7, 8). Sodium thiosulfate is a key ingredient employed in clinical antidote therapy for cyanide poisoning. However, the actual sulfur donor compounds used by the enzyme *in vivo* are not known, but might include cysteine persulfide, thiotaurine, thiocystine, among other possibilities (S. Baskin, personal communication); (c) The levels of rhodanese are very high in the liver and kidney (4), suggesting a role in detoxification of xenobiotics, such as cyanide; (d) The nasal epithelia, which represents a likely site for the absorption of aerosolized cyanogenic compounds or HCN, also contains high levels of this enzyme (9, 10); And (e) a significant portion of rhodanese molecules are localized within the mitochondrial matrix. One can speculate that intra-mitochondrial rhodanese protects against cyanide-mediated damage to cytochrome oxidase, which is involved in electron transport.

The primary amino acid sequence and 3-D structure of bovine rhodanese purified from liver have been determined (11, 12). The enzyme is composed of a single polypeptide chain consisting of two globular domains, A and B, of equivalent size (approximately 140 amino acid residues each), separated by a short connecting hinge region located on the exterior surface of the A domain (11-13). The active site of bovine rhodanese is contained within the B domain, where Cys-248 (often referred to as Cys-247 elsewhere in the literature, when the initiating methionine is excluded) acts as an intermediate acceptor of the sulfane sulfur atom from a donor cofactor. This covalently-bound sulfur atom within the enzyme intermediate is then transferred to the substrate (e.g., CN⁻). Little is known regarding the contribution of the A domain in catalysis, although the amino terminus contained within the A domain is responsible for chaperonin-mediated transport of the enzyme into the mitochondrial matrix (14).

We have previously isolated and sequenced the cDNA encoding rat liver rhodanese (15). The protein encoded by the open reading frame (ORF) specifies 297 amino acid residues and is approximately 92 percent identical to the well characterized bovine enzyme (11), which has also been cloned (16). When conservative amino acid substitutions are included, the rat and cow enzymes are 98% similar, thus very highly conserved (15). Using the rat rhodanese cDNA as a probe, we have determined the gene maps to mouse chromosome 15 (17). A portion of the human rhodanese cDNA has been recently obtained (18). Another cDNA has been reported to represent the human rhodanese gene (19). However, the latter isolate is unlikely to be the authentic human rhodanese gene based on the lack of sequence similarity to the other known mammalian rhodanese genes. The activity and identity of this rhodanese-related cDNA clone (19) is uncertain.

In view of the power of molecular genetic studies in mice and our desire to develop the mouse as a model species for studies of rhodanese molecular biology and cyanide toxicity, we have cloned the mouse rhodanese cDNA. In this article we report the mouse rhodanese cDNA and primary amino acid sequences, and demonstration of enzymatic activity from the mouse and rat genes when expressed in *E. coli*.

MATERIALS AND METHODS: All general molecular biology methods used in this work are described elsewhere (15, 20, 21), unless stated otherwise. The ampicillin-resistant bacterial colonies were cultured in LB or 2xYT media + 50 µg/ml ampicillin, and plasmid DNAs were

prepared by alkaline lysis, phenol-chloroform extraction, and precipitation in ethanol and sodium acetate. Oligonucleotide primers were synthesized commercially (Genosys, Houston, TX):

pUC Forward 5'-CGCCAGGGTTTTCCAGTCACGAC
 pUC Reverse 5'-TCACACAGGAAACAGCTATGAC
 gt 11 Forward 5'-GGTGGCGACGACTCCTGGAGCCCG
 MRN 105 5'-GAATTCTCAGGCCTTCCCACTCTTC
 Rho Nco 5'-GGGTACCGAGCTTGAATTCGCCATGGTGCATCAGGTGCTC
 SN 117 5'-GCCACCATGGTACATCAGGTGCTC
 SN 114 5'-GGTCCAGCTGGTGATTG

Additional sequencing primers were developed based on the derived rodent cDNA sequences.

Cloning of mouse rhodanese cDNA: The BALB/c male mouse liver cDNA library was obtained from Clontech Inc. (Paolo Alto, CA). The lambda gt 11 library was grown in Y1090r cells and amplified by inoculation of 1.0 ml saturated cultures (in LB containing 0.2% maltose) with 2 ul of phage stocks and absorption for 15 min at 37°C. The 1.0 ml cultures were added to 50 ml of medium (LB containing 10 mM MgSO₄) and incubated in a shaker for 8 hr. Then, DNA samples were prepared of the amplified phage library stocks by alkaline lysis.

PCR amplification was performed on samples (ca. 30 ng) of the amplified phage DNA preparations with a Perkin-Elmer Cetus DNA Thermocycler, AmpliTaq DNA Polymerase (Perkin-Elmer, Norwalk, CT), and oligonucleotide primers complementary to the phage vector and the rhodanese cDNA insert (gt 11 Forward and MRN 105, respectively). The reaction conditions included a Perkin-Elmer Cetus PCR Core Reagent Kit and the following cycles: a single initial melting at 94°C for 4 minutes, denaturation at 94°C for 2 minutes, annealing at 58°C for 2 minutes, extension at 72°C for 2 minutes, for a total of 30 cycles, followed by an additional 7 minutes at 72°C. All PCR amplicon fragments were ligated into pT7Blue(R) Vector (Novagen Inc., Madison WI), transformed into *E. coli* Nova Blue cells, and plated onto LB agar plates containing 0.8 mg/ml IPTG, 0.8 mg/ml X-gal, and 50 µg/ml ampicillin. White transformants (i.e., β-galactosidase negative) containing inserts were grown in LB or 2xYT media containing 50 µg/ml of ampicillin. The final full-length clone, pSN369, contains the entire open reading frame, including the translation start and stop codons for mouse rhodanese. To verify the DNA sequence (below), additional cDNA isolates were obtained via PCR from this library (Clontech) and another mouse liver library (Stratagene, La Jolla, CA).

The 3' untranslated portion of mouse rhodanese cDNA was obtained by PCR amplification using the same commercial mouse liver cDNA library. DNA samples of the amplified library were PCR amplified with SN 114 and gt11 Forward primers and similar conditions. The longest amplicon fragment (0.5 kb) was subcloned into pT7 Blue vector, to yield clone pSN2037 containing regions corresponding to the carboxyl terminus and the 3' untranslated region.

NcoI-modified 5'-end of mouse and rat cDNAs: In order to create an NcoI site at the Met initiation codon at the 5' end of the mouse cDNA, pSN369 was reamplified by PCR using SN 117 and pUC Reverse primers and the following conditions: initial denaturing at 95°C for 5 min, then 30 cycles of denaturing at 95°C for 3 min, annealing at 40°C for 3 min, and extension at 72°C for 3 min, followed by a final additional extension at 72°C for 7 min. The amplicon fragment was subcloned into pT7 Blue (R) vector (Novagen, Madison, WI) to yield clone pTM1193. The artificially-produced NcoI site provides a convenient asymmetric restriction cleavage site for the determination of orientation, and provides an improved eucaryotic ribosome binding site for translational initiation.

The rat rhodanese cDNA previously isolated from a Lambda gt11 liver cDNA library, termed pRhoD (subcloned into pGEM-3Z vector), lacks the presumptive initiating methionine¹ and valine² codons at the N-terminus (15). We have subsequently repaired the cDNA so that the N-terminus specifies a Met¹Val² dipeptide fused in frame and also contains an NcoI restriction enzyme site (specifying a eucaryotic ribosome binding site for the initiation of translation). This construct, pRhoN, was created by polymerase chain reaction (PCR) site-directed mutagenesis of pRhoD, using synthetic oligonucleotides to the rhodanese N-terminus (Rho Nco) and the vector T7 promoter primer, and AmpliTaq DNA polymerase (Perkin Elmer Cetus). The amplified 1.0 kb fragment was then cut with EcoRI, electrophoresed on agarose, excised from the gel, and electroeluted. The fragment was then ligated into EcoRI-cut pUC19 to yield pRhoN.

DNA sequencing: The nucleotide sequences of the full-length mouse *Tsr* cDNA (pSN369), the mouse 3' end portion (pSN2037), and the 'NcoI-modified' cDNA clones (mouse-derived pTM1193 and rat-derived pRhoN) were determined by double-stranded, dideoxynucleotide chain-

termination DNA sequencing using the 7-deaza-dGTP Sequencing Kit (USB, Cleveland, OH), appropriate oligonucleotide primers (both vector- and rhodanese-derived), and [α - 32 P]-dATP (NEN, Boston, MA). Double stranded DNA was denatured in 1.0 M NaOH at room temperature for 10 min, followed by pH adjustment with 0.3 M sodium acetate and ethanol precipitation. The reactions were resolved on 6 - 8% polyacrylamide plus 50% urea gel electrophoresis, followed by overnight exposure of Fuji RX film. The sequence was obtained for both strands of DNA and was analyzed on a Macintosh IIsi computer using the MacVector 4.5 software (Eastman-Kodak, Rochester, NY) and the NCBI Entrez version 15.0 database on CD ROM.

The GenBank database accession number for the full-length mouse rhodanese cDNA (composite sequence derived from pSN369 and pSN2037) is U35741. The EMBL database accession number for rat rhodanese cDNA (15) is X56228, and the GenBank accession number for bovine rhodanese cDNA (16) is M58561.

Mouse and rat rhodanese cDNA expression constructs: We prepared three full-length rhodanese cDNA plasmid vectors for expression in bacteria by fusing the 0.9 kb EcoRI fragment of mouse pSN369 into pUC19, and the 0.9 kb and 1.0 kb NcoI-EcoRI fragments of the mouse pTM1193 and rat pRhoN cDNAs, respectively, into NcoI-EcoRI cut pKK388-1 (Pharmacia). Orientation of the pSN369 cDNA fragment insert was determined by SmaI digestion. In each case, we 'engineered' the position of the bacterial ribosome binding sites within 10 - 20 bp from the ATG (Met¹ initiator) codon.

pSN361: The pSN361 construct consists of the EcoRI mouse cDNA fragment from pSN369 cloned in the sense orientation within the pUC19 vector. This construct produces rhodanese as an artificial operon having a partial *lacZ* gene immediately 5' to the mouse rhodanese cDNA cloned into the EcoRI site. The bacterial *lac* promoter is inducible by IPTG. Coincidentally, pSN369 is the antisense version of pSN361.

pTM2098: IPTG-induced expression of the NcoI-repaired mouse cDNA is accomplished by this construct, consisting of the NcoI-EcoRI fragment of pTM1193 subcloned into NcoI-EcoRI cut pKK388-1 vector (Pharmacia). This construct utilizes the IPTG-inducible *trp/lac* fusion promoter, referred to as either *tac* or *trc*.

pSN1049: The NcoI-EcoRI fragment of rat pRhoN was subcloned into NcoI-EcoRI cut pKK388-1 vector yielding the pSN1049 construct.

Determination of recombinant rhodanese activities following transformation of the constructs into *E. coli* JM109 cells: Competent *E. coli* strain JM109 cells were transformed with pSN361, pTM2098, pSN1049, pUC19 (vector), pKK388-1 (vector), and pSN369 (antisense mouse cDNA), and grown overnight to saturation at 37°C in LB or 2xYT cultures containing 50 μ g/ml ampicillin. These cultures were used to inoculate additional tubes +/- 0.4 mg/ml IPTG and 10 nM sodium thiosulfate. IPTG induces transcription of recombinant rhodanese cDNA genes from the *lac* or *tac* promoters.

E. coli cell extracts were prepared from the pellets of 1.0 ml of cells grown either 6 hr or overnight (16+ hr), freezing in a dry ice-ethanol bath for 2 min, followed by 8 min in an ice water bath (22). The cell pellets were freeze-thaw cycled for a total of three times. Then 0.1 ml of 20 mM Tris HCl pH 8.6 was added, the samples were briefly vortexed, and placed on ice for 30 min. The samples were centrifuged and the aqueous extracts (< 0.1 ml) excluding cellular debris (i.e., pellet) were removed to separate tubes on ice.

Samples of cell extracts and controls [the blank contained Tris alone, and purified bovine rhodanese (Sigma) served as a positive control] were assayed using a spectrophotometric method (2). The extract samples (routinely 20 μ l each) were added to tubes containing 1.0 ml assay mixture (40 mM potassium phosphate, 50 mM potassium cyanide, 50 mM sodium thiosulfate) preincubated at 37°C. The samples were incubated for precisely 15 min and stopped by the addition of 0.5 ml 15% formaldehyde. The reactions were then added to 1.5 ml ferric nitrate reagent (8.7% nitric acid, 165 mM ferric nitrate), and spectrophotometric measurements were performed at OD₄₆₀. The level of expression of each construct was determined in multiple assays by dividing the OD₄₆₀ of the extract of the expression construct in the presence of IPTG by the negative control vector lacking a cDNA insert (i.e., pUC19 or pKK388-1).

RESULTS: Cloning and Sequencing of Mouse Rhodanese cDNA: The full-length mouse rhodanese cDNA, obtained by PCR amplification of a mouse liver cDNA library, is demonstrated in Figure 1. An open reading frame (ORF) encoding 297 amino acid residues was identified.

```

90
*
GCAGAACTGACACTATGGTACATCAGGTGC TCTACCGAGCGCTGGTATCCACCAAGTGGC TGGCGGAATCCATTGGTCTGGCCGCTGG
M V H Q V L Y R A L V S T K W L A E S I R S G R L>
180
*
GGCCCCAGCCTTCGGGTGCTGGACGCGTCTT GGTACTCACCCGGGACCCGCCAGGCAUGCA AAGAGTACCAGGAGCGACACGTGCCTGGCG
G P S L R V L D A S W Y S P G T R Q A R K E Y Q E R H V P G>
270
*
CGTCCTTCTTTGATATAGAGGAATGCCGGG ACACGACTTCGCCTTATGAGATGATGCTGC CCAGTGAGGCCCACTTTGGGGACTATGTGG
A S F F D I E E C R D T T S P Y E M M L P S E A H F G D Y V>
360
*
GCAACCTGGGCATAAGCAACGACACACATG TGGTGGTATACGATGGAGATGACCTGGGCA GCTTCTATGCACCCAGAGTCTGGTGGATGT
G N L G I S N D T H V V V Y D G D D L G S F Y A P R V W W M>
450
*
TTCTGTGTTTGGCCACCGCACAGTGTGAG TGCTCAATGGTGGCTTCCGGAAGTGGCTGA AGGAGGGCCACCCGGTGACATCTGAGCCCT
F R V F G H R T V S V L N G G F R N W L K E G H P V T S E P>
540
*
CGAGGCCAGAGCCAGCCGTTTCAAAGCCA CACTGAATCTCTCCCTGCTCAAGACCTATG AGCAGGTCTAGAGAATCTCCAGTCTAAAA
S R P E P A V F K A T L N L S L L K T Y E Q V L E N L Q S K>
630
*
GGTTCAGCTGGTGGACTCTCGGGCCCAAG GACGGTACCTGGGCACACAACCGGAGCCGG ATATAGTAGGACTAGACTCGGGCCACATCC
R F Q L V D S R A Q G R Y L G T Q P E P D I V G L D S G H I>
720
*
GTGGCTCAGTCAACATGCCCTTCATGGACT TCCTGACGAAAGATGGCTTCGAGAAGAGCC CAGAGGAGCTGCGTGCCATATTCCAGGACA
R G S V N M P F M D F L T K D G F E K S P E E L R A I F Q D>
810
*
AGAAGGTGGACCTCTCACAGCCCCTCATTG CCACGTGCCGCAAGGGGGTCACTGCCTGTC ACGTTGCCCTGGCTGCCTACCTTTGTGGCA
K K V D L S Q P L I A T C R K G V T A C H V A L A A Y L C G>
900
*
AACCCGATGTGGCTGTTTACGATGGCTCCT GGTGAGAGTGGTTCGCGAGGGCACCCCCAG AGACCCGTGTGTCCAGGGGAAGAGTGGGA
K P D V A V Y D G S W S E W F R R A P P E T R V S Q G K S G>
990
*
AGGCCTGAGTGTGTGACGCTCCTCCTCTG CCCACCAATCCAGCCAGTTCCTTCAGGG AGGTTGGTGTGACCTGCTGTAGGTTACCT
K A *
TTTAGGCTACGGATATTGACAGGGTT

```

Figure 1. The full-length cDNA and amino acid sequences of mouse rhodanese obtained by manual dideoxynucleotide sequencing. The *Sma*I and *Kpn*I sites are located at positions 129 and 574 of the 1016 bp composite cDNA. The amino acid residues of the ORF are indicated using the single letter code.

Analysis of the ORF by MacVector (version 4.5) software predicted that unprocessed nascent mouse rhodanese exhibits a mass of 33,464 Daltons and a pI of 8.18. This pI estimate is identical to rat rhodanese, but is considerably higher (more basic) than for the bovine enzyme (pI 6.84). In addition to the coding region, 14 bp of untranslated mRNA were identified at the 5' end and 108 bp were obtained at the 3' end.

The primary amino acid sequences of rhodanese based on cloned full-length cDNAs from mouse, rat (15), and cow (16) are presented in Figure 2. The 5'-end of the rat cDNA was not



Figure 2. Rhodanese amino acid sequence comparison for mouse (this work), rat (15), and cow (16), based on the open reading frames of cloned cDNAs: Amino acid substitutions are indicated using the single letter code for the ORF-derived sequences of rat and cow relative to mouse. The amino terminal Met¹Val² dipeptide of rat is presumed. The rat gene contains a unique Met²¹¹ to Val²¹¹ substitution. The active site in all three genes is specified by Cys²⁴⁸.

available in our previously published rat pRhoD cDNA (15), so it was created by PCR amplification to include a presumed Met¹-Val² dipeptide at the amino terminus in the full-length pRhoN clone, consistent with the other known enzymes. The mouse amino acid sequence is 97.3% identical to rat and 89.9% identical to cow, whereas rat is slightly closer (91.9%) to cow. At the DNA level, mouse is 94.6% identical to rat and 86.3% identical to cow within the coding regions. Comparison of the amino acid substitutions between the two rodent species revealed eight differences, seven of which were conserved between rat and cow. A single discriminating mutation results in a Met²¹¹ to Val²¹¹ substitution within the rat gene, which was not present in either mouse or cow.

Expression of mouse and rat rhodanese cDNAs in *E. coli*: In order to demonstrate biochemical activities by the enzymes encoded by the mouse and rat rhodanese cDNAs, fragments containing both ORFs were subcloned into IPTG-inducible bacterial expression vectors. The cyanide:thiosulfate sulfurtransferase activities were determined for extracts from *E. coli* JM109 containing the mouse (pSN361 and pTM2099) and rat (pSN1049) cDNA expression constructs, and appropriate negative control vectors (Table I). The mouse recombinant protein exhibited approximately a 5 x increase (mean) in activity following 6 hours of IPTG induction, in general, and approximately a 7 x increase (mean) in activity following overnight (16 hr+) induction, relative to vector control-transformed JM109 cells. The mouse recombinant enzyme extracts were active when assayed from 20 to 51°C, with optimal activity at 37°C (data not shown). Mouse rhodanese within the extracts was inactivated at 63°C or when treated with the protease inhibitor,

phenylmethylsulfonylfluoride (PMSF). Inclusion of a nonionic detergent, 1% IGEPAL (CA-630; Sigma), did not affect activity. By comparison, the rat enzyme extracts exhibited similar increases in activity when IPTG treated relative to vector-transformed control JM109 cells (Table I). Since activity from the expressed rat rhodanese cDNA has not been previously reported, these findings demonstrate that both the mouse and rat cDNAs encode functional products.

DISCUSSION: The cloned rhodanese cDNAs for mouse and rat exhibited cyanide-metabolizing activity when expressed in *E. coli*, indicating that substantial posttranslational modification is not required for catalytic activity of this enzyme. Comparison of the nascent proteins based on their cDNA ORFs to the primary amino acid sequence of purified bovine liver rhodanese (11, 12) suggests that a mammalian carboxypeptidase may process three residues (Gly²⁹⁵Lys²⁹⁶Ala²⁹⁷) at the C-terminus of the protein *in vivo*. The unprocessed mouse and rat enzymes have a predicted isoelectric point (pI) of 8.18, which is considerably higher than for cow (pI 6.84). It is not known whether this pI difference results in any alterations of subcellular localization or kinetic, catalytic, or other biochemical properties. Sequence alignment between the three species also revealed a discriminating mutation of Met²¹¹ to Val²¹¹ within the rat gene. The Val²¹¹ is located in the B domain within 1 nm of the active site Cys²⁴⁸ (15), but does not inactivate the enzyme.

The mouse amino acid sequence is 97.3% identical to rat and 89.9% identical to cow. The two rodent genes differ at only 8 of 297 residues (2.7% nonidentity). This relatively low level of sequence difference among the two rodents and between rodents and an ungulate mammal is expected, and is consistent with our assertion that all three genes are homologs of one another.

TABLE I
Recombinant Rhodanese Expression in *E. coli*

cDNA	Vector	Culture	IPTG-Induced Expression Level		
		Time (hrs)	(mean)	(range)	(n)
Mouse	pUC19	6	4.95 x	(1.9 - 7.6) x	7
		16+	7.13 x	(5.2 - 8.6) x	4
Mouse	pKK388-1	6	9.73 x	(2.8 - 16.7) x	2
		16+	3.31 x	-	1
Rat	pKK388-1	6	4.06 x	(2.2 - 7.9) x	14
		16+	8.32 x	(1.7 - 16.5) x	5

Enzymatic activity of recombinant mouse and rat rhodanese in extracts from *E. coli* JM109 expressing each cDNA under the control of an IPTG-inducible promoter: Cells containing the expression constructs and appropriate vector controls were incubated in culture at 37°C for 6 or 16+ hr in the absence or presence of IPTG and sodium thiosulfate. Multiple extracts were then assayed for cyanide-metabolizing activity using a spectrophotometric method (2). The levels, ranges, and number of experiments of IPTG-induced expression relative to vector controls are summarized. Individual variations may be due to differences in growth rates, final density of each culture, and to preparation of the extracts.

This is further validated by the catalytic activities of the expressed cDNAs from the three species (this work; 16). We have previously reported a similar three-species sequence alignment with the structurally-unrelated calpactin I light chain genes, recently termed as S100A10 (21). Substitutions were observed at 7 of 97 residues (7.2% nonidentity) between mouse and rat S100A10 genes, thus at a higher level of dissimilarity than expected for two rodents. These results suggest that rodent rhodanese genes are more highly conserved than are the S100A10 genes.

The mouse rhodanese gene, *Tst*, has been previously mapped to chromosome 15 by us using an interspecific backcross mapping panel (17). The recent repositioning of one of the *Tst*-linked DNA markers in this region has resulted in the reassignment of *Tst* to approximately 45-46 cM (17 *erratum*), relative to the *Chromosome 15 Consensus Map* (23). This location is within a region of mouse chromosome 15 that is homologous to human chromosome 22q. The human homologs of several flanking (centromeric) mouse genes *Pva*, *Bzrp*, and *Dial* have been mapped to human 22q12 - qter (23, 24), and a portion of the human *TST* gene has been utilized to map rhodanese to human chromosome 22 using human-rodent somatic cell hybrids (18). Thus, we predict that human *TST* is contained within 22q12 - qter. At present, no known genetic diseases have been identified that are associated with alterations in rhodanese activity, or for which rhodanese is a positional candidate gene on chromosomes 22 in humans or 15 in mice. The rat *Tst* gene, which we have previously cloned at the cDNA level (15), likely maps to rat chromosome 7 based on syntenic homology to this region of mouse chromosome 15 (25-27).

The mouse rhodanese cDNA may now be used in a variety of experimental studies, including site-directed mutagenesis, transgenic and knock-out mice, and overexpression of recombinant protein. The purified recombinant form may be intravenous infused into mice to test whether allotypic rhodanese can be used therapeutically in cases of acute cyanide poisoning. By comparison, erythrocyte-encapsulated rhodanese, purified from liver, can confer heightened cyanide-metabolizing activity *in vivo* (28). Since we have obtained the mouse rhodanese cDNA, these studies are now feasible and could further establish the mouse as a molecular genetic model for cyanide toxicity.

ACKNOWLEDGMENTS: This work was supported by the U.S. Army Medical Research, Development, Acquisition and Logistics Command under Contract No. DAMD17-94-C-4044 (TPD). The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation. The authors are grateful to Dr. S. Baskin (U.S. Army Medical Research Institute of Chemical Defense) for critically reading the manuscript, to A. Miranda (SFBR) for technical assistance, and to Dr. E. Leiter, M. Bridgett, H. Chapman, and L. Rowe (The Jackson Laboratory) for collaborative assistance concerning rhodanese gene mapping. The GenBank accession number for mouse rhodanese cDNA is U35741.

REFERENCES

1. Sorbo, B.H. (1953) *Acta Chem. Scand.* 7:1129-1136.
2. Westley, J. (1981) *Meth. Enzymol.* 77:285-291.
3. Jarabak, R. (1981) *Meth. Enzymol.* 77:291-297.
4. Aminlari, M., Gilanpour, H., Taghavianpour, H., Veseghi, T. (1989) *Comp. Biochem. Physiol.* 92C:259-262.
5. Cerletti, P. (1986) *Trends in Biochem. Sci.* 11:369-372.
6. Sandberg, W., Graves, M.C., Rabinowitz, J.C. (1987) *Trends in Biochem. Sci.* 12:56.

7. Johnson, W.S., Hall, A.H., Rumack, B.H. (1989) *Am. J. Emergency Med.* 7:437-440.
8. Schultz, V. (1984) *Clin. Pharmacokinetics* 9:239-251.
9. Lewis, J.L., Rhoades, C.E., Gervasi, P.-G., Griffith, W.C., Dahl, A.R. (1991) *Toxicol. Appl. Pharmacol.* 18:114-120.
10. Lewis, J.L., Rhoades, C.E., Bice, D.E., Harkema, J.R., Hotchkiss, J.A., Sylvester, D.M., Dahl, A.R. (1992) *Anat. Record* 232:620-627.
11. Russell, J., Weng, L., Keim, P.S., Heinrichson, R.L. (1978) *J. Biol. Chem.* 253:8102-8108.
12. Ploegman, J.H., Drent, G., Kalk, K.H., Hol, W.G.J., Heinrichson, R.L., Keim, P., Weng, L., Russell, J. (1978) *Nature* 273:124-129.
13. Hol, W.G.J. (1987) *Biol. Macromol. Assem.* 3:483-522.
14. Hammen, P.K., Gorenstein, D.G., Weiner, H. (1994) *Biochemistry* 33, 8610-8617.
15. Weiland, K.L., Dooley, T.P. (1991) *Biochem. J.* 275:227-231.
16. Miller, D.M., Delgado, R., Chirgwin, J.M., Hardies, S.C., Horowitz, P.M. (1991) *J. Biol. Chem.* 266:4686-4691.
17. Leiter, E.H., Chapman, H.D., Dooley, T.P. (1993) *Mouse Genome* 91:567-568, and *Erratum* in (1995) *Mouse Genome* 93:863.
18. Durkin, A.S., Niernan, W.C., Zoghbi, H., Jones, C., Kozak, C.A., Maglott, D.R. (1994) *Cytogenet. Cell Genet.* 65:86-91.
19. Pallini, R., Guazzi, G.C., Cannella, C., Cacace, M.G. (1991) *Biochem. Biophys. Res. Com.* 180:887-893.
20. Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* 2nd Edition (Cold Spring Harbor Laboratory Press, New York).
21. Dooley, T.P., Weiland, K.L., Simon, M. (1992) *Genomics* 13:866-868.
22. Johnson, B.H., Hecht, M.H. (1994) *BioTechnology* 12:1357-1360.
23. Mock, B.A., Eppig, J.T., Neumann, P.E., Huppi, K.E. (1994) *Mamm. Genome* 5:s217-s228.
24. Bell, C.J., Budarf, M.L., Nieuwenhuijsen, B.W., Barnoski, B.L., Buetow, K.H., Campbell, K., Colbert, A.M.E., Collins, J., Daly, M., Desjardins, P.R., DeZwaan, T., Eckman, B., Foote, S., Hart, K., Hiester, K., Van Het Hoog, M.J., Hopper, E., Kaufman, A., McDermid, H.E., Overton, G.C., Reeve, M.P., Searls, D.B., Stein, L., Valmiki, V.H., Watson, E., Williams, S., Winston, R., Nussbaum, R.L., Lander E.S., Fischbeck, K.H., Emanuel, B.S., Hudson, T.J. (1995) *Hum. Molec. Genet.* 4:59-69.
25. Jacob, H.J., Brown, D.M., Bunker R.K., Daly, M.J., Dzau, V.J., Goodman, A., Koike, G., Kren, V., Kurtz, T., Lernmark, A., Levan, G., Mao, Y.-P., Pettersson, A., Pravanec, M., Simon, J.S., Szpirer, C., Szpirer, J., Trollet, M.R., Winer, E.S., Lander, E.S. (1995) *Nature Genet.* 9:63-69.
26. Levan, G., Szpirer, J., Szpirer, C., Klinga, K., Hanson, C., Islam, M.-Q. (1991) *Genomics* 10:699-718.
27. Yamada, J., Kuramoto, T., Serikawa, T. (1994) *Mamm. Genome* 5:63-83.
28. Leung, P., Cannon, E.P., Petrikovics, I., Hawkins, A., Way, J.L. (1991) *Toxicol. Appl. Pharmacol.* 110:268-274.